AD_____

GRANT NO: DAMD17-94-J-4221

TITLE: Breast Cancer: Involvement of Epidermal Growth Factor Receptor, MDM2 and P53 Mutations

PRINCIPAL INVESTIGATOR(S): Doctor John H. Ludes-Meyers

CONTRACTING ORGANIZATION: The University of Texas
Health Science Center at San Antonio
San Antonio, Texas 78284-7758

REPORT DATE: October 1995

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

2

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	October 1995	Annual 15 Sep 94 - 14 Sep 95

4. TITLE AND SUBTITLE
Breast Cancer: Involvement of Epidermal Growth Factor Receptor, MDM2 and P53 Mutations

5. FUNDING NUMBERS
DAMD17-94-J-4221

6. AUTHOR(S)
Dr. John H. Ludes-Meyers

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The University of Texas
Health Science Center at San Antonio
San Antonio, Texas 78284-7758

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

The epidermal growth factor receptor (EGFR) is a transmembrane glycoprotein with an extracellular epidermal growth factor (EGF)-binding domain and an intracellular protein tyrosine kinase domain. EGF stimulates cell proliferation by directly activating EGFR, leading to signal transduction through an intracellular cascade of second messengers. It was previously demonstrated that the wild-type human p53 tumor suppressor can transactivate the human EGFR promoter through a p53-response element mapped to the region from -569 to -104, relative to the translational start site. Further analysis has identified the 27-base pair region from -265 to -239 as the p53-binding site/response element. This region possesses sequence homology to a p53-binding site/response element in the human retinoblastoma susceptibility (Rb) gene promoter, and is responsive to wild-type p53 when cloned upstream of a minimal heterologous promoter. Mobility-shift and DNase I footprinting assays indicated that wild-type p53 binds directly to the response element. The p53-binding site/response element overlaps the binding sites for both positive (ETF) and negative (GCF) regulators of EGFR promoter activity, and contains or borders the most 5' of the EGFR *in vivo* transcriptional start sites.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Breast Cancer, p53, Epidermal Growth Factor Receptor	30
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

GENERAL INSTRUCTIONS FOR COMPLETING SF 298

The Report Documentation Page (RDP) is used in announcing and cataloging reports. It is important that this information be consistent with the rest of the report, particularly the cover and title page. Instructions for filling in each block of the form follow. It is important to ***stay within the lines*** to meet ***optical scanning requirements***.

Block 1. Agency Use Only *(Leave blank)*.

Block 2. Report Date. Full publication date including day, month, and year, if available (e.g. 1 Jan 88). Must cite at least the year.

Block 3. Type of Report and Dates Covered. State whether report is interim, final, etc. If applicable, enter inclusive report dates (e.g. 10 Jun 87 - 30 Jun 88).

Block 4. Title and Subtitle. A title is taken from the part of the report that provides the most meaningful and complete information. When a report is prepared in more than one volume, repeat the primary title, add volume number, and include subtitle for the specific volume. On classified documents enter the title classification in parentheses.

Block 5. Funding Numbers. To include contract and grant numbers; may include program element number(s), project number(s), task number(s), and work unit number(s). Use the following labels:

C	- Contract	**PR**	- Project
G	- Grant	**TA**	- Task
PE	- Program Element	**WU**	- Work Unit Accession No.

Block 6. Author(s). Name(s) of person(s) responsible for writing the report, performing the research, or credited with the content of the report. If editor or compiler, this should follow the name(s).

Block 7. Performing Organization Name(s) and Address(es). Self-explanatory.

Block 8. Performing Organization Report Number. Enter the unique alphanumeric report number(s) assigned by the organization performing the report.

Block 9. Sponsoring/Monitoring Agency Name(s) and Address(es). Self-explanatory.

Block 10. Sponsoring/Monitoring Agency Report Number. *(If known)*

Block 11. Supplementary Notes. Enter information not included elsewhere such as: Prepared in cooperation with...; Trans. of...; To be published in.... When a report is revised, include a statement whether the new report supersedes or supplements the older report.

Block 12a. Distribution/Availability Statement. Denotes public availability or limitations. Cite any availability to the public. Enter additional limitations or special markings in all capitals (e.g. NOFORN, REL, ITAR).

- **DOD** - See DoDD 5230.24, "Distribution Statements on Technical Documents."
- **DOE** - See authorities.
- **NASA** - See Handbook NHB 2200.2.
- **NTIS** - Leave blank.

Block 12b. Distribution Code.

- **DOD** - Leave blank.
- **DOE** - Enter DOE distribution categories from the Standard Distribution for Unclassified Scientific and Technical Reports.
- **NASA** - Leave blank.
- **NTIS** - Leave blank.

Block 13. Abstract. Include a brief *(Maximum 200 words)* factual summary of the most significant information contained in the report.

Block 14. Subject Terms. Keywords or phrases identifying major subjects in the report.

Block 15. Number of Pages. Enter the total number of pages.

Block 16. Price Code. Enter appropriate price code *(NTIS only)*.

Blocks 17. - 19. Security Classifications. Self-explanatory. Enter U.S. Security Classification in accordance with U.S. Security Regulations (i.e., UNCLASSIFIED). If form contains classified information, stamp classification on the top and bottom of the page.

Block 20. Limitation of Abstract. This block must be completed to assign a limitation to the abstract. Enter either UL (unlimited) or SAR (same as report). An entry in this block is necessary if the abstract is to be limited. If blank, the abstract is assumed to be unlimited.

*U.S.GPO:1993-0-358-779

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the US Army.

____ Where copyrighted material is quoted, permission has been obtained to use such material.

____ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

__X__ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

____ In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

__X__ For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

__X__ In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

__X__ In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

____ In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

John H. Sanders Major 10/11/95

PI - Signature Date

TABLE OF CONTENTS

LIST OF FIGURES

Introduction

Mutation of the p53 gene is, at present, the most frequently reported genetic defect in human cancer. While normally present at very low levels owing to its short half-life, in response to DNA damage p53 accumulates to high levels that effect either a G_1 cell cycle arrest or apoptosis (Maltzman and Czyzyk, 1984; Kastan *et al.*, 1991; Kuerbitz *et al.*, 1992; Lin *et al.*, 1992; Lowe *et al.*, 1993). A G_1 arrest would presumably allow time for DNA repair to occur prior to entry into S phase and DNA replication. The genetic damage that might otherwise initiate tumorigenesis is thereby either repaired or eliminated. Wild-type p53 has also been shown to be a sequence-specific DNA-binding protein (Kern *et al.*, 1991) that can activate transcription from promoters containing p53-binding sites (Kern *et al.*, 1992), presumably by virtue of its association with the TATA-binding protein (TBP) (Seto *et al.*, 1992; Liu *et al.*, 1993; Martin *et al.*, 1993; Chen *et al.*, 1993; Deb, S. P. *et al.*, 1994). The vast majority of p53 mutations are single amino acid substitutions within the domain responsible for sequence-specific DNA-binding and mutant proteins are defective at this property (Kern *et al.*, 1991). p53 might therefore mediate tumor suppression by activating the expression of genes involved in apoptosis, cell cycle regulation, or DNA repair.

One such gene expresses the major p53-induced transcript detected in a system where a wild-type p53 gene which had been placed under the regulated control of a hormone response element (El-Deiry *et al.*, 1993). The gene encodes a 21-kDa protein (p21, WAF-1, or CIP1), that acts as an inhibitor of cyclin/cyclin-dependent kinase (Cdk) complexes (Harper *et al.*, 1993). The G_1 cell cycle arrest that wild-type p53 generates in response to DNA damage may therefore be mediated by an inhibition of the cyclin D/Cdk and cyclin E/Cdk complexes needed for progression into S phase. p53 has also been shown to activate expression of the growth arrest and DNA damage-inducible gene, *GADD45* (Kastan *et al.*, 1992).

While p21 may directly mediate the p53-dependent G_1 arrest that occurs in response to DNA damage, evidence for an effector of p53-dependent apoptosis has been provided with the observation that p53 can activate the expression of Bax, a heterodimeric partner and dominant inhibitor of Bcl-2 (Miyashita *et al.*, 1994). Bcl-2 had previously been shown to block p53-dependent apoptosis (Wang *et al.*, 1993b), and the ratio of Bcl-2 to Bax may determine the relative susceptibility of cells to stimuli that induce apoptosis (Oltvai *et al.*, 1993).

p53 also activates the expression of thrombospondin-1 (TSP-1), an inhibitor of angiogenesis (Dameron *et al.*, 1994). Angiogenesis is the generation of new capillaries from existing venules, and solid tumors, which must develop a vasculature in order to grow and metastasize efficiently, are potently angiogenic. Wild-type p53 might therefore mediate tumor suppression by inhibiting this essential feature of neoplastic progression.

Given its pivotal role in tumor suppression, it is not altogether surprising that p53 may also regulate its own expression and activity. In what appears to be both positive and negative autoregulatory mechanisms, p53 activates expression of its own gene (Deffie *et al.*, 1993), and the *mdm2* oncogene (Barak *et al.*, 1993; Wu *et al.*, 1993). The MDM2 oncoprotein binds to the N-terminal transcriptional activation domain of p53 and inhibits p53-mediated transactivation (Momand *et al.*, 1992; Brown *et al.*, 1993; Oliner *et al.*, 1993).

While p53-mediated transactivation of p21, GADD45, Bax, TSP-1, p53 and MDM2 expression has been verified, the search continues for other genes that may be activated by p53. It was previously demonstrated that wild-type human p53 can transactivate the human EGFR promoter in a dose-dependent manner in Saos-2 cells (Deb, S. P. *et al.*, 1994). EGFR is a transmembrane glycoprotein with an extracellular EGF-binding domain and an intracellular protein tyrosine kinase domain. EGF stimulates cell proliferation by directly activating EGFR, leading to signal transduction through an intracellular cascade of second messengers. A p53-response element on the EGFR promoter was mapped by deletion analysis to the region from -569 to -104, relative to the translational start site (Deb, S. P. *et al.*, 1994). In this study further analysis has identified the 27-base pair region from -265 to -239 as the p53-binding site/response

element. This region may be particularly relevant to the regulation of EGFR transcription as it overlaps the binding sites for both positive (ETF) (Kageyama *et al.*, 1988) and negative (GCF) (Kageyama and Pastan, 1989) regulators of EGFR promoter activity, and contains the most 5' of the EGFR *in vivo* transcriptional start sites (Ishii *et al.*, 1985; Haley *et al.*, 1987).

Materials and Methods

DNA plasmids. The p53-cWT expression plasmid contains a wild-type human p53 cDNA under the regulation of the human cytomegalovirus (CMV) immediate-early promoter in the pCMV-Bam expression vector (Hinds *et al.*, 1990; Subler *et al.*, 1992) (donated by Arnold J. Levine). The series of N- and C-terminal deletion derivatives of wild-type human p53 was generated as described (Subler *et al.*, 1994).

The chloramphenicol acetyltransferase (CAT) plasmids utilized contain the *Escherichia coli* CAT gene under the transcriptional control of human EGFR promoter sequences. The 3' end of each of the following EGFR-CAT constructs (Johnson *et al.*, 1988; donated by Glenn T. Merlino) is at -20, relative to the EGFR translational start site, while the 5' ends map to the following restriction sites and positions: pERCAT-1 (*Hin*d III, -1109); pERCAT-4 (*Stu* I, -911); pERCAT-6 (*Pvu* II, -771); pERCAT-7 (*Taq* I, -569); pERCAT-8 (*Dde* I, -490); pERCAT-9 (*Ava* II, -389); and pERCAT-15 (*Ava* I, -104). pERCAT-1 (Δ -404 to -152) is an internal promoter deletion of the region between two *Not* I sites in pERCAT-1. A second set of 5'-deletion derivatives was generated by BAL-31 exonuclease digestion to -320, -276, and -246.

TATA-CAT constructs were generated by cloning human EGFR or Rb promoter sequences into the *Bgl* II site upstream of the adenovirus major late promoter TATA element in TATA-CAT (Wang and Gralla, 1991). EGFR (-265 to -239), EGFR-MUT (-265 to -239), and Rb (-88 to -63) utilized synthetic oligonucleotides with the following sequences: EGFR (-265 to -239), 5'-AGCTAGACGTCCGGGCAGCCCCCGGCG-3'; EGFR-MUT (-265 to -239), 5'-AGCTAGACGTCCGTGAAGCACACGGCG-3'; Rb (-88 to -63), 5'-AGCTAGAGGACGGG GCGTGCCCCGACGTGC-3'. EGFR (-320 to -228) was generated by BAL-31 deletion and *Not* I digestion.

Cell culture and transfection. Human osteosarcoma (Saos-2) cells were cultured, and transfected by the calcium phosphate-DNA coprecipitation method, as described previously (Subler *et al.*, 1992).

Chloramphenicol acetyltransferase assays. CAT assays were performed, and assay results analyzed, as described previously (Subler *et al.*, 1992).

Mobility-shift and DNase I footprinting assays. Wild-type and mutant (281G) human p53s were expressed by recombinant baculoviruses and purified by immunoaffinity chromatography, as described previously (Martin *et al.*, 1993).

Results

Localization of a wild-type p53-response element within a 285-base pair segment of the EGFR promoter. To determine the region of the human EGFR promoter responsive to wild-type p53, we tested a series of 5' promoter deletions which utilized restriction sites at -911, -771, -569, -490, -389, and -104 (Johnson *et al.*, 1988; donated by Glenn T. Merlino). Figure 1 represents CAT assays performed after cotransfection of Saos-2 cells with 2.5 μg of one of the 5'-deletion derivatives, along with 5 μg of either the pCMV-Bam expression vector or the wild-type human p53 expression plasmid. Whereas wild-type p53 activated all 5' deletions to -389, it failed to activate the 5' deletion to -104. These results suggest that a p53-response element is located within the 285-bp region of the EGFR promoter between -389 and -104.

Mapping of the p53-response element to a 27-base pair region of the human EGFR promoter. To determine whether the initial mapping of the p53-response element by 5'-deletion analysis of the EGFR promoter may have failed to detect additional upstream p53-response elements, an internal promoter deletion (between the -404 and -152 *Not* I sites of pERCAT-1, Figure 2) was constructed. As Figure 3A indicates, deletion of this region almost completely eliminates wild-type p53-mediated transactivation, suggesting that this region is required for transactivation.

To further define the p53-response element, a second set of 5'-deletion derivatives was generated by BAL-31 exonuclease digestion of the EGFR promoter to -320, -276, and -246 (Figure 2). As Figure 3B indicates, deletion of the region between -276 and -246 almost

completely eliminates transactivation by wild-type p53, suggesting that this region may contain the p53-response element. Upon comparison of the -276 to -246 region of the EGFR promoter with known p53-binding sites, sequence homology between a p53-binding site/response element in the human Rb gene promoter (-88 to -63) and the EGFR sequence from -265 to -239 was noted (Osifchin *et al.*, 1994) (Figure 4A). Moreover, nine of the ten G residues in the human Rb promoter defined by methylation interference analysis as important for p53 binding are conserved in the corresponding region of the human EGFR promoter (Figure 4A). The Rb sequence had previously been shown to function as a p53-response element when cloned upstream of the E1B TATA element. To determine whether the homologous region (-265 to -239) of the human EGFR promoter could function similarly, an oligonucleotide containing this region was cloned upstream of the adenovirus major late promoter TATA element in the TATA-CAT construct (Wang and Gralla, 1991). As controls, the corresponding Rb sequence (-88 to -63), the EGFR region from -320 to -228 (generated by BAL-31 deletion and *Not* I digestion), and a mutated version of the EGFR region from -265 to -239 (EGFR-MUT), were all cloned into TATA-CAT. EGFR-MUT contains four G→T and C→A transversions at positions corresponding to Rb residues defined by methylation interference analysis as important for p53 binding. As Figure 4B indicates, the region of the human EGFR promoter from -265 to -239 was responsive to wild-type p53 in transfected Saos-2 cells, while the mutations in EGFR-MUT completely eliminated wild-type p53-mediated transactivation.

Domain requirements for wild-type p53-mediated transcriptional activation of the human EGFR promoter. To determine the domains on the wild-type protein required for p53-mediated transactivation of the human EGFR promoter, a series of N- and C-terminal deletion derivatives was generated as described (Subler *et al.*, 1994). Saos-2 cells were cotransfected with 2.5 μg of pERCAT-1 (containing the EGFR promoter from -1109 to -20) along with 5 μg of a plasmid expressing either the wild-type human p53 protein or one of its N- or C-terminal deletion derivatives (or with 5 μg of the pCMV-Bam expression vector). The results in Figure 5 indicate that whereas the N-terminal 59 amino acids of p53 (previously shown to bind to TBP, Liu *et al.*,

1993) are required for transactivation of pERCAT-1, the C-terminal 67 residues (containing most of the p53 oligomerization domain) are not required. This corresponds to the domain requirements for wild-type p53-mediated transactivation of PG_{13}-CAT, a synthetic promoter containing p53-binding sites (Kern *et al.*, 1992; Subler *et al.*, 1994), and suggests that sequence-specific DNA-binding is required for wild-type p53-mediated transactivation of the human EGFR promoter.

p53 binds directly to its EGFR promoter response element. To determine whether p53 could bind directly to its EGFR promoter response element, a mobility-shift assay was performed with baculovirus-expressed and immunoaffinity-purified wild-type human p53 (Martin *et al.*, 1993) and a radiolabeled oligonucleotide containing the EGFR promoter sequence from -265 to -239. Figure 6 (lane 1) indicates the presence of a retarded complex that was eliminated by the addition of specific competitor (RGC, lanes 2 and 3), but not by addition of a nonspecific competitor (MUT, lanes 4 and 5). The presence of p53 in the retarded complex was verified by addition of the p53-specific monoclonal antibody DO-1, which further retarded the complex (Figure 6, lane 6); addition of a control antibody (EGFR, lane 7) had no effect on the p53/DNA complex.

DNase I footprinting with a radiolabeled DNA fragment containing the EGFR promoter sequence from -320 to -228 indicated that purified wild-type human p53 protected the region from -266 to -241, as well as a second shorter region from -237 to -231 (Figure 7A, lanes 2 and 3). Purified mutant p53-281G did not generate a footprint (Figure 7A, lanes 4 and 5). No additional areas of protection by wild-type p53 were detected on the larger region from -490 to -20 (pERCAT-8, data not shown), indicating complete correspondence between *in vitro* DNA-binding and *in vivo* transcriptional activation assays in the determination of the p53-binding site/response element on the EGFR promoter. Sequence analysis also revealed that the protected region from -266 to -244 is actually a degenerate consensus p53-binding site, with 3-bp spacing between two decameric 5'-RRRC(A/T)(T/A)GYYY-3' repeats and two base substitutions per repeat (Figure 7B). Deviation from the consensus sequence (El-Deiry *et al.*, 1992) has been

noted previously for other p53-binding sites (El-Deiry *et al.*, 1993; Wu *et al.*, 1993; Zambetti and Levine, 1993). The mutations in EGFR-MUT that eliminated wild-type p53-mediated transactivation (Figure 4B) also eliminated four of the eight conserved (with respect to the consensus sequence) bases in the 3' decamer (Figure 7B), and presumably DNA-binding as a result, since two decamers are required for binding (El-Deiry *et al.*, 1992).

Conclusions

Growth factors modulate cell growth and differentiation via interaction with their receptors. Among the growth factors that have been widely studied, epidermal growth factor (EGF) stimulates cell proliferation by directly activating its receptor (EGFR) leading to signal transduction through an intracellular cascade of second messengers (Carpenter and Cohen, 1990; Fantl *et al.*, 1993). EGFR is a transmembrane glycoprotein of 170 kDa with an extracellular EGF-binding domain and an intracellular protein tyrosine kinase domain (reviewed in Fantl *et al.*, 1993). EGF, phorbolesters, dibutyryl cyclic AMP, dexamethasone, thyroid hormone, and retinoic acid have all been shown to activate the human EGFR promoter (Clark *et al.*, 1985; Hudson *et al.*, 1989). The involvement of several controlling mechanisms suggests that regulation of EGFR transcription may be critically important for normal cell growth and proliferation. Our recent observation that several tumor-derived human p53 mutants can significantly activate the human EGFR promoter (Deb, S. P. *et al.*, 1994) may be relevant to the overexpression of EGFR that has been detected in a variety of tumors and transformed cell lines.

In that communication we also reported that wild-type human p53 can transactivate the human EGFR promoter in a dose-dependent manner. The p53-response element on the EGFR promoter was mapped by deletion analysis to the region from -569 to -104, relative to the translational start site. The analysis described here has now identified the 27-base pair region from -265 to -239 as the p53-response element. Cotransfection of Saos-2 cells with a second set of 5'-deletion derivatives of the EGFR promoter generated by BAL-31 digestion (to -320, -276, and -246) indicated that the region between -276 and -246 may contain the p53-response element (Figure 3B). Comparison of the -276 to -246 region of the EGFR promoter with known p53-binding sites revealed sequence homology between a p53-binding site/response element in the human Rb gene promoter (-88 to -63) and the EGFR sequence from -265 to -239 (Figure 4A) (Osifchin *et al.*, 1994). Furthermore, nine of the ten G residues in the human Rb promoter defined by methylation interference analysis as important for p53 binding are conserved in the

corresponding region of the human EGFR promoter (Figure 4A). The Rb sequence had previously been shown to function as a p53-response element when cloned upstream of the E1B TATA element. To determine whether the homologous region (-265 to -239) of the human EGFR promoter could function similarly, an oligonucleotide containing this region was cloned upstream of the adenovirus major late promoter TATA element in the TATA-CAT construct (Wang and Gralla, 1991). As Figure 4B indicates, the region of the human EGFR promoter from -265 to -239 was responsive to wild-type p53 in transfected Saos-2 cells, while four G→T and C →A transversions at positions corresponding to Rb residues defined by methylation interference analysis as important for p53 binding completely eliminated wild-type p53-mediated transactivation. This region may be particularly relevant to the regulation of EGFR transcription as it overlaps the binding sites for both positive (ETF, -250 to -235) (Kageyama *et al.*, 1988) and negative (GCF, -280 to -265 and -245 to -227) (Kageyama and Pastan, 1989) regulators of EGFR promoter activity, and contains or borders the most 5' of the EGFR *in vivo* transcriptional start sites (at -266 and -260) (Ishii *et al.*, 1985; Haley *et al.*, 1987).

To determine the domains on the wild-type protein required for p53-mediated transactivation of the human EGFR promoter, a series of N- and C-terminal deletion derivatives was utilized (Subler *et al.*, 1994). Cotransfection of Saos-2 cells with pERCAT-1 (containing EGFR promoter sequences from -1109 to -20) and the p53 deletion derivatives indicated that the N-terminal 59 amino acids of p53 (previously shown to bind to the TATA-binding protein) are required for transactivation of pERCAT-1, while the C-terminal 67 residues (containing most of the p53 oligomerization domain) are not required (Figure 5). The C-terminal deletion to amino acid 277, deleting part of conserved domain V, eliminated p53-mediated EGFR promoter transactivation. This is presumably due to the loss of sequence-specific DNA-binding, as the domain responsible for this function (residues 91-307) encompasses conserved domains II through V (Pavletich *et al.*, 1993; Wang *et al.*, 1993; Bargonetti *et al.*, 1993). These requirements correspond to the domain requirements for wild-type p53-mediated transactivation

of PG_{13}-CAT, a synthetic promoter containing p53-binding sites (Kern *et al.*, 1992; Subler *et al.*, 1994), and suggest that wild-type p53 may bind its EGFR response element directly.

To determine whether p53 could bind directly to its EGFR promoter response element, a mobility-shift assay was performed with baculovirus-expressed and immunoaffinity-purified wild-type human p53 (Martin *et al.*, 1993) and a radiolabeled oligonucleotide containing the EGFR promoter sequence from -265 to -239. Figure 6 (lane 1) indicates the presence of a retarded complex, that was eliminated by the addition of specific competitor (RGC, lanes 2 and 3), but not by addition of a nonspecific competitor (MUT, lanes 4 and 5). The presence of p53 in the retarded complex was verified by addition of the p53-specific monoclonal antibody DO-1, which further retarded the complex (Figure 6, lane6); addition of a control antibody (EGFR, lane7) had no effect on the p53/DNA complex.

DNase I footprinting with a radiolabeled DNA fragment containing the EGFR promoter sequence from -320 to -228 indicated that purified wild-type human p53 protected the region from -266 to -241, as well as a second shorter region from -237 to -231 (Figure 7A, lanes 2 and 3). Purified mutant p53-281G did not generate a footprint (Figure 7A, lanes 4 and 5). No additional areas of protection by wild-type p53 were detected on the larger region from -490 to -20 (pERCAT-8, data not shown), indicating complete correspondence between *in vitro* DNA-binding and *in vivo* transcriptional activation assays in the determination of the p53-binding site/response element on the EGFR promoter. Sequence analysis also revealed that the protected region from -266 to -244 is actually a degenerate consensus p53-binding site, with 3-bp spacing between two decameric 5'-RRRC(A/T)(T/A)GYYY-3' repeats and two base substitutions per repeat (Figure 7B). Deviation from the consensus sequence (El-Deiry *et al.*, 1992) has been noted previously for other p53-binding sites (El-Deiry *et al.*, 1993; Wu *et al.*, 1993; Zambetti and Levine, 1993). The mutations in EGFR-MUT that eliminated wild-type p53-mediated transactivation (Figure 4B) also eliminated four of the eight conserved (with respect to the consensus sequence) bases in the 3' decamer (Figure 7B), and presumably DNA-binding as a result, since two decamers are required for binding (El-Deiry *et al.*, 1992).

As mentioned earlier, the high levels of wild-type p53 that accumulate in response to DNA damage can effect either a G_1 cell cycle arrest or programmed cell death (apoptosis) (Maltzman and Czyzyk, 1984; Kastan *et al.*, 1991; Kuerbitz *et al.*, 1992; Lin *et al.*, 1992; Lowe *et al.*, 1993). p53 is therefore considered to be a negative regulator of cell proliferation. However, earlier studies using p53 antibodies or antisense p53 RNA indicated that wild-type p53 may be required for cell proliferation (Mercer *et al.*, 1984; Shohat *et al.*, 1987). To confirm the significance of p53-mediated EGFR promoter transactivation, it will be necessary to demonstrate increased EGFR mRNA and protein levels upon p53 overexpression. In any event, as EGFR is known to acquire oncogenic properties when its structure or regulation becomes disrupted (Haley *et al.*, 1987; Stoscheck and King, 1986; Velu *et al.*, 1987), transactivation by wild-type p53 may represent a novel mechanism of cell growth regulation.

Future work will focus on determining the role of wild-type p53 transactivation of the endogenous EGFR promoter and further characterization of mutant p53 transactivation of the EGFR promoter (Deb, S.P. et.al., 1994).

REFERENCES

Barak, Y., T. Juven, R. Haffner, and M. Oren. 1993. MDM2 expression is induced by wild-type p53 activity. EMBO J. 12: 461-468.

Bargonetti, J., J.J. Manfredi, X. Chen, D. Marshak, and C. Prives. 1993. A proteolytic fragment from the central region of p53 has marked sequence specific DNA binding activity when generated from wild type but not from oncogenic mutant p53 protein. Genes Dev. 7: 2565-2574.

Brown, D.R., S. Deb, R.M. Muñoz, M.A. Subler, and S.P Deb. 1993. The tumor suppressor p53 and the viral oncoprotein SV40 T antigen bind to overlapping domains on the MDM2 protein. Mol. Cell. Biol. 13: 6849-6857.

Carpenter, G. and S. Cohen. 1990. Epidermal growth factor. J. Biol. Chem. 265: 7709-7712.

Chen, X., G. Farmer, H. Zhu, R. Prywes, and C. Prives. 1993. Cooperative DNA-binding of p53 with TFIID (TBP): a possible mechanism for transcriptional activation. Genes Dev. 7: 1837-1849.

Clark, A.J.L., S. Ishi, N. Richert, G.T. Merlino, and I. Pastan. 1985. Epidermal growth factor regulates the expression of its own receptor. Proc. Natl. Acad. Sci. USA 82: 8374-8378.

Dameron, K.M., O.V. Volpert, M.A. Tainsky, and N. Bouck. 1994. Control of angiogenesis in fibroblasts by p53 regulation of thrombospondin-1. Science 265: 1582-1584.

Deb, S.P., R.M. Muñoz, D.R. Brown, M.A. Subler, and S. Deb. 1994. Wild-type human p53 activates the human epidermal growth factor receptor promoter. Oncogene 9: 1341-1349.

Deffie, A., H. Wu, V. Reinke, and G. Lozano. 1993. The tumor suppressor p53 regulates its own transcription. Mol. Cell. Biol. 13: 3415-3423.

El-Deiry, W.S., S.E. Kern , J.A. Pietenpol, K.W. Kinzler, and B. Vogelstein. 1992. Definition of a consensus binding site for p53. Nature Genet. 1: 45-49.

El-Deiry, W.S., T. Tokino, V.E. Velculescu, D.B. Levy, R. Parsons, J.M. Trent, D. Lin, W.E. Mercer, K.W. Kinzler, and B. Vogelstein. 1993. WAF1, a potential mediator of p53 tumor suppression. Cell 75: 817-825.

Fantl, W.J., D.E. Johnson, and L.T. Williams. 1993. Signaling by receptor tyrosine kinases. Annu. Rev. Biochem. 62: 453-481.

Haley, J., N. Whittle, P. Bennet, D. Kinchington, A. Ulrich, and M. Waterfield. 1987. The human EGF receptor gene: structure of the 110 kb locus and identification of sequences regulating its transcription. Oncogene Res. 1: 375-396.

Harper, J.W., G.R. Adami, N. Wei, K. Keyomarsi, and S.J. Elledge. 1993. The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G_1 cyclin-dependent kinases. Cell 75: 805-816.

Hinds, P.W., C.A. Finlay, R.S. Quartin, S.J. Baker, E.R. Fearon, B. Vogelstein, and A.J. Levine. 1990. Mutant p53 DNA clones from human colon carcinomas cooperate with *ras* in transforming primary rat cells: a comparison of the "hot spot" mutant phenotypes. Cell Growth Differ. 1: 571-580.

Hudson, L.G., J.B. Santon, and G.N. Gill. 1989. Regulation of epidermal growth factor receptor gene expression. Mol. Endocrinol. 3: 400-408.

Ishii, S., Y.-H. Xu, R.H. Stratton, B.A. Roe, G.T. Merlino, and I. Pastan. 1985. Characterization and sequence of the promoter region of the human epidermal growth factor receptor gene. Proc. Natl. Acad. Sci. USA 82: 4920-4924.

Johnson, A.C., S. Ishi, Y. Jinno, I. Pastan, and G.T. Merlino. 1988. Epidermal growth factor receptor gene promoter. Deletion analysis and identification of nuclear protein binding sites. J. Biol. Chem. 263: 5693-5699.

Kageyama, R., G.T. Merlino, and I. Pastan. 1988. A transcription factor active on the epidermal growth factor receptor gene. Proc. Natl. Acad. Sci. USA 85: 5016-5020.

Kageyama, R., and I. Pastan. 1989. Molecular cloning and characterization of a human DNA binding factor that represses transcription. Cell 59: 815-825.

Kastan, M.B., O. Onyekwere, D. Sidransky, B.Vogelstein, and R.W. Craig. 1991. Participation of p53 protein in the cellular response to DNA damage. Cancer Res. 51: 6304-6311.

Kastan, M.B., Q. Zhan, W.S. El-Deiry, F. Carrier, T. Jacks, W.V. Walsh, B.S. Plukett, B. Vogelstein, and A.J. Fornace. 1992. A mammalian cell cycle checkpoint pathway utilizing p53 and GADD45 is defective in ataxia-telangiectasia. Cell 71: 587-597.

Kern, S.E., K.W. Kinzler, A. Bruskin, D. Jarosz, P. Friedman, C. Prives, and B. Vogelstein. 1991. Identification of p53 as a sequence-specific DNA-binding protein. Science 252: 1708-1711.

Kern, S.E., J.A. Pietenpol, S. Thiagalingam, A. Seymour, K.W. Kinzler, and B. Vogelstein. 1992. Oncogenic forms of p53 inhibit p53 regulated gene expression. Science 256: 827-830.

Kuerbitz, S.J., B.S. Plunkett, W.V. Walsh, and M.B. Kastan. 1992. Wild-type p53 is a cell cycle checkpoint determinant following irradiation. Proc. Natl. Acad. Sci. USA 89: 7491-7495.

Lin. D., M. Shields, S. Ulrich, E. Appella, and W. Mercer. 1992. Growth arrest induced by wild-type p53 protein blocks cells prior to or near the restriction point in late G_1 phase. Proc. Natl. Acad. Sci. USA 89: 9210-9214.

Liu, X., C.W. Miller, P.H. Koeffler, and A.J. Berk. 1993. The p53 activation domain binds the TATA box-binding polypeptide in holo-TFIID, and a neighboring p53 domain inhibits transcription. Mol. Cell. Biol. 13: 3291-3300.

Lowe, S.W., E.M. Schmitt, S.W. Smith, B.A. Osborne, and T. Jacks. 1993. p53 is required for radiation-induced apoptosis in mouse thymocytes. Nature 362: 847-849.

Maltzman, W., and L. Czyzyk. 1984. UV irradiation stimulates levels of p53 cellular tumor antigen in nontransformed mouse cells. Mol. Cell. Biol. 4: 1689-1694.

Martin, D.W., R.M. Muñoz, M.A. Subler, and S. Deb. 1993a. p53 binds to the TATA-binding protein-TATA complex. J. Biol. Chem. 268: 13062-13067.

Mercer, W.E., C. Avignolo, and R. Baserga. 1984. Role of the p53 protein in cell proliferation as studied by microinjection of monoclonal antibodies. Mol. Cell. Biol. 4: 276-281.

Miyashita, T., S. Krajewski, M. Krajewska, H.G. Wang, H.K. Lin, D.A. Liebermann, B. Hoffman, and J.C. Reed. 1994. Tumor suppressor p53 is a regulator of bcl-2 and bax gene expression *in vitro* and *in vivo*. Oncogene. 9: 1799-1805.

Momand, J., G.P. Zambetti, D.C. Olson, D. George, and A.J. Levine. 1992. The mdm-2 oncogene product forms a complex with the p53 protein and inhibits p53-mediated transactivation. Cell 69: 1237-1245.

Oliner, J.D., J.A. Pietenpol, S. Thiagalingam, J. Gyuris, K.W. Kinzler, and B. Vogelstein. 1993. Oncoprotein MDM2 conceals the activation domain of tumour suppressor p53. Nature 362: 857-860.

Oltvai, Z.N., C.L. Milliman, and S.J. Korsmeyer. 1993. Bcl-2 heterodimerizes *in vivo* with a conserved homolog, Bax, that accelerates programmed cell death. Cell 74: 609-619.

Osifchin, N.E., D. Jiang, N. Ohtani-Fujita, T. Fujita, M. Carroza, S.-J. Kim, T. Sakai, and P.D. Robbins. 1994. Identification of a p53 binding site in the human retinoblastoma susceptibility gene promoter. J. Biol. Chem. 269: 6383-6389.

Pavletich, N.P., K.A. Chambers, and C. Pabo. 1993. The DNA binding domain of p53 contains the four conserved regions and major mutation hot spots. Genes Dev. 7: 2556-2564.

Seto, E., A. Usheva, G.P. Zambetti, J. Momand, N. Horikoshi, R. Weinmann, A.J. Levine, and T. Shenk. 1992. Wild- type p53 binds to the TATA-binding protein and represses transcription. Proc. Natl. Acad. Sci. USA 89: 12028-12032.

Shohat, O.M., Greenberg, D. Reisman, M. Oren, and V. Rotter. 1987. Inhibition of cell growth mediated by plasmids encoding p53 anti-sense. Oncogene 1: 277-283.

Stoscheck, C.M., and L.E. King. 1986. Role of epidermal growth factor in carcinogenesis. Cancer Res. 46: 1030-1037.

Subler, M.A., D.W. Martin, and S. Deb. 1992. Inhibition of viral and cellular promoters by human wild-type p53. J. Virol. 66: 4757-4762.

Subler, M.A., D.W. Martin, and S. Deb. 1994b. Overlapping domains on the p53 protein regulate its transcriptional activation and repression functions. Oncogene 9: 1351-1359.

Velu, T.J, L. Beguinot, W.C.Vass., M.C. Willingham, G.T. Merlino, I. Pastan, and D.R. Lowy. 1987. Epidermal growth factor dependent transformation by a human EGF receptor proto-oncogene. Science 238: 1408-1410.

Wang, W., and J.D. Gralla. 1991. Differential ability of proximal and remote element pairs to cooperate in activating RNA polymerase II transcription. Mol. Cell. Biol. 11: 4561-4571.

Wang, Y., M. Reed, P. Wang, J.E. Stenger, G. Mayr, M.E. Anderson, J.F. Schwedes, and P. Tegtmeyer. 1993a. p53 domains: identification and characterization of two autonomous DNA binding regions. Genes Dev. 7: 2575-2586.

Wang, Y., L. Szekely, I. Okan, G. Klein, and K. G. Wiman. 1993b. Wild-type p53-triggered apoptosis is inhibited by *bcl*-2 in a v-*myc*-induced T-cell lymphoma line. Oncogene 8: 3427-3431.

Wu, X., J.H. Bayle, D. Olson, and A.J. Levine. 1993. The p53-mdm-2 autoregulatory feedback loop. Genes Dev. 7: 1126-1132.

Zambetti, G.P., and A.J. Levine. 1993. A comparison of the biological activities of wild-type and mutant p53. FASEB J. 7: 855-865.

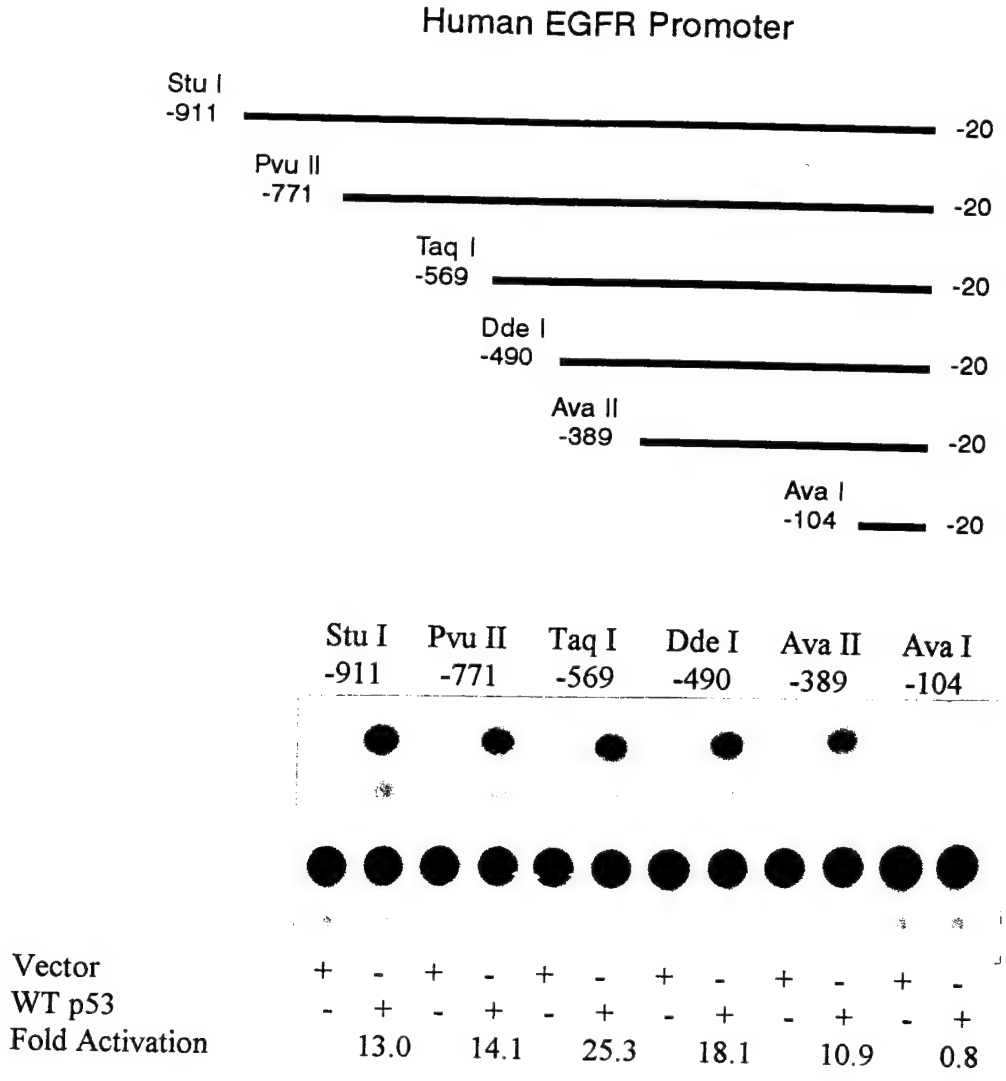

Figure 1. Localization of a wild-type p53-response element within a 285-base pair segment of the human EGFR promoter. The top panel represents the series of 5'-deletion derivatives of the EGFR promoter utilized. The restriction sites used to generate the deletions are indicated. The bottom panel consists of CAT assays performed after cotransfection of Saos-2 cells with the indicated EGFR promoter-CAT constructs, along with either the pCMV-Bam expression vector or the wild-type human p53 expression plasmid. The results indicate the presence of a p53-response element within the region from -389 to -104.

```
     -544 GGGAC CGGGTCCAGA GGGGCAGTGC TGGGAACGCC CCTCTCGGAA -500
           Ava II

-499 ATTAACTCCT CAGGGCACCC GCTCCCCTCC CATGCGCCGC CCCACTCCCC -450
              Dde I

-449 CGGAGACTAG GCCCGCGGGG GCCACCGCTG TCCACCGCCT CCGGCggcCG -400
                                                    Not I

-399 CTGGCCTTGG GTCCCCGCTG CTGGTTCTCC TCCCTCCTCC TCGCATTCTC -350
               Ava II
                                      -320
                                       >
-349 CTCCTCCTCT GCTCCTCCCG ATCCCTCCTC CGCCGCCTGG TCCCTCCTCC -300

                                -276        -266   -260
                                  >           ↓      ↓
-299 TCCCGCCCTG CCTCCCcGCG CCTCGGCCCG CGCGAGCTAG ACGTCCGGGC -250
                                                 Rb homology
      -246
        >
-249 AGCCCCCGGC GCAGCGCGGC CGCAGCaGCC TCCGCCCCCC GCACGGTGTG -200
     Rb homology        Not I

-199 AGCGCCCGCC gCGgCCGAGG CGGCCGGAGT CCCGAGCTAG CCCCGGCGGC -150
                                                    Not I

-149 cGCCGCCGCC CAGACCGGAC GACAGGCCAC CTCGTCgGCG TCCGCCCGAG -100
                                                    Ava I

 -99 TCCCCGCCTC GCCGCCAACG CCACAACCAC CGCGCACGGC CCCCTGACTC -50

 -49 CGTCCAGTAT TGATCGGGAG AGCCGGAGCG -20
```

Figure 2. DNA sequence of the human EGFR promoter from -544 to -20, relative to the translational start site. *Ava* II, *Dde* I, *Not* I, and *Ava* I restriction sites are shown. Letters in small case indicate bases absent from a previously published sequence, which also had a T (rather than C) at position -255 (Johnson *et al.*, 1988a). Arrowheads at -320, -276, and -246 represent the 5' ends of the promoter derivatives generated by BAL-31 deletion. Arrows at -266 and -260 represent the most 5' of the EGFR *in vivo* transcriptional start sites (Ishii *et al.*, 1985; Haley *et al.*, 1987). The region from -265 to -239 possessing homology to the p53-binding site/response element in the human Rb promoter is underlined.

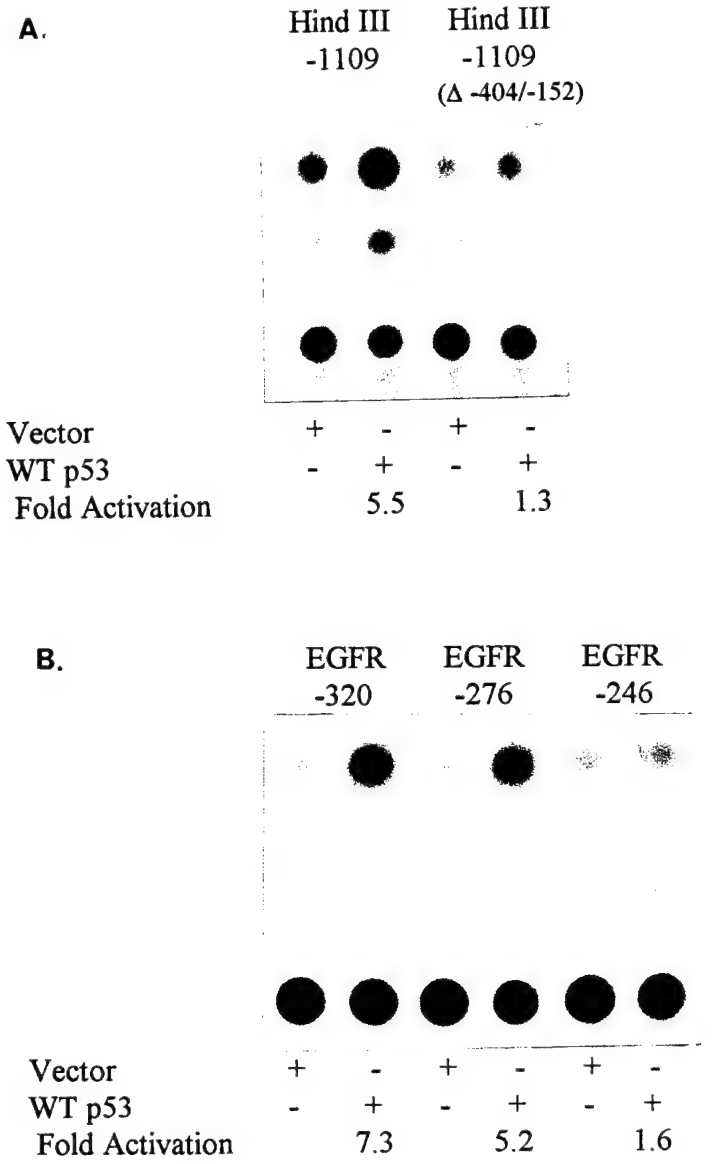

Figure 3. Finer mapping of the p53-response element on the human EGFR promoter. A. Internal EGFR promoter deletion. An internal promoter deletion of the region between the -404 and -152 *Not* I sites of pERCAT-1 almost completely eliminates wild-type p53-mediated transactivation, demonstrating that this region is required for transactivation. **B. BAL-31 5' EGFR promoter deletions.** Cotransfection of Saos-2 cells with a second set of 5'-deletion derivatives of the EGFR promoter generated by BAL-31 digestion (to -320, -276, and -246) indicates that the region between -276 and -246 may contain the p53-response element. The 3' end of each promoter derivative is at -20.

```
                                  t a   a a
EGFR    -265 AGCTAGACGTCCGGGCAGCCCCCGGCGCAG -236
             ******* * * ****   ***** **
Rb           AGCTAGAGGACGGGGCGTGCCCCGACGTGC -63
```

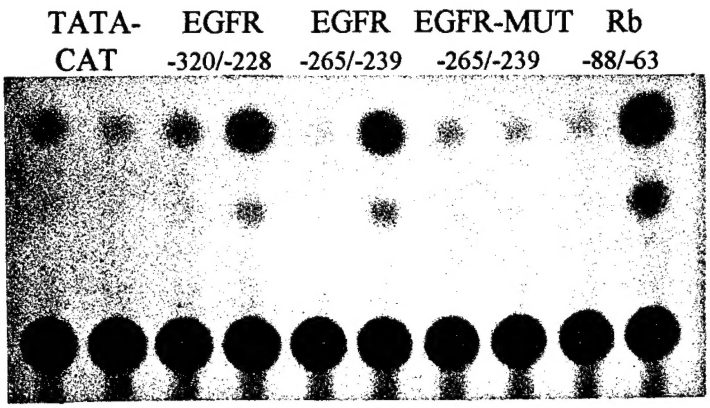

Figure 4. (A) Sequence comparison of the EGFR and Rb promoter p53-response elements. Asterisks indicate sequence identity. Arrowheads point to the 10 G (or C on complementary strand) residues of the Rb promoter p53-response element determined by methylation interference to be important for p53 binding. Letters in small case indicate the G→T and C→A mutations in the EGFR-MUT construct. **(B) The 27-base region (from -265 to -239) of the human EGFR promoter is responsive to wild-type p53 when cloned upstream of a TATA element.** To determine whether the EGFR promoter region from -265 to -239 (possessing sequence homology with a p53-binding site/response element in the human Rb promoter) could function as a p53-response element when cloned upstream of a minimal heterologous promoter, an oligonucleotide containing this region was cloned upstream of the adenovirus major late promoter TATA element in the TATA-CAT construct (Wang and Gralla, 1991). As controls, the corresponding Rb sequence (-88 to -63), the EGFR region from -320 to -228 (generated by BAL-31 deletion and *Not* I digestion), and a mutated version of the EGFR region from -265 to -239 (EGFR-MUT), were all cloned into TATA-CAT. Cotransfection of Saos-2 cells with 5 μg of either the pCMV-Bam expression vector or the wild-type p53 expression plasmid, along with 2.5 μg of either TATA-CAT or one of its derivatives indicated that the region of the human EGFR promoter from -265 to -239 is responsive to wild-type p53.

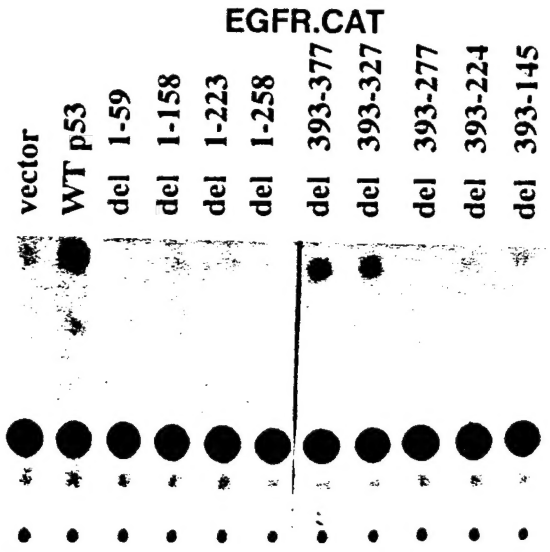

Figure 5. Domain requirements for wild-type p53-mediated transcriptional activation of the human EGFR promoter. To determine the domains on the wild-type protein required for p53-mediated transactivation of the human EGFR promoter, a series of N- and C-terminal deletion derivatives was generated as described (Subler *et al.*, 1994b). Saos-2 cells were cotransfected with 2.5 μg of pERCAT-1 (containing the EGFR promoter from -1109 to -20) along with 5 μg of a plasmid expressing either the wild-type human p53 protein or one of its N- or C-terminal deletion derivatives (or with 5 μg of the pCMV-Bam expression vector). The domains required for p53-mediated transactivation of the human EGFR promoter correspond to those required for transactivation of PG_{13}-CAT a synthetic promoter containing p53-binding sites (Kern *et al.*, 1992; Subler *et al.*, 1994b).

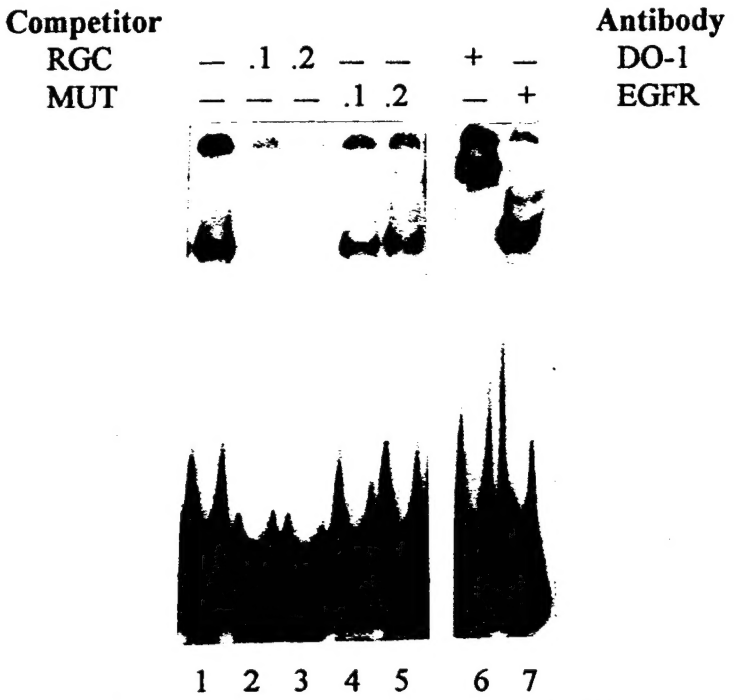

Figure 6. Wild-type human p53 binds to the human EGFR promoter. The EGFR promoter (-263 to -234) was labeled with $\gamma[^{32}P]$ ATP and was incubated with immunopurified p53 as described in Materials and Methods. The protein-DNA complexes were resolved in a native 5% polyacrylamide gel and autoradiographed. All lanes received equal amounts of immunopurified p53. Lane 1 was without competitor; lanes 2 and 3 were with 100 and 200 ng of double-stranded oligonucleotides corresponding to wild-type p53-binding site (Kern *et.al.*, 1992) as described in Materials and Methods, lanes 3 and 4 were with 100 and 200 ng of double-stranded oligonucleotides with mutated binding sites, lane 6 was with 1 ul of anti-p53 antibody (DO-1, Santa Cruz Biotech. Inc.) and lane 7 was with 1 ul of a nonspecific monoclonal antibody (anti-EGFR antibody, Oncogene Science). EMSA indicates that the oligonucleotides with wild-type p53-binding site sequence competed but not those with mutant sequences indicating that the binding is sequence specific.

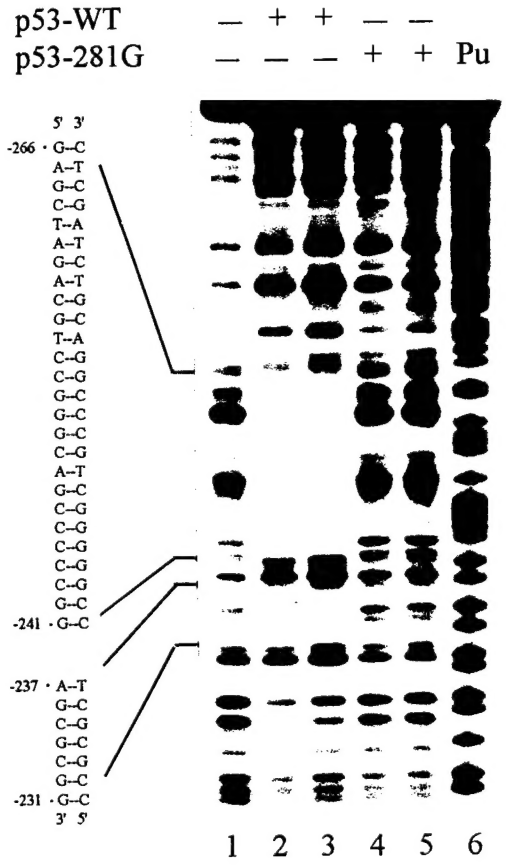

Figure 7. DNase I footprint of human wild-type p53 on the human EGFR promoter. DNase I footprinting was carried out with the immunopurified wild-type p53 and mutant p53-281G as described in Materials and Methods. Lane 1 recieved 100 ng BSA, lanes 2 and 3 were with 50 and 100 ng of immunopurified wild-type human p53, lanes 4 and 5 were with immunopurified p53-281G protein. The G+A sequencing ladder was carried out in parallel and is represented by nucleotide positions on the left. Wild-type p53 protects sequences from -266 to -231.